Mitochondrial and Nuclear Localization of a Novel Pea Thioredoxin: Identification of Its Mitochondrial Target Proteins

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Plants contain several genes encoding thioredoxins (Trxs), small proteins involved in the regulation of the activity of many enzymes through dithiol-disulfide exchange. In addition to chloroplastic and cytoplasmic Trx systems, plant mitochondria contain a reduced nicotinamide adenine dinucleotide phosphate-dependent Trx reductase and a specific Trx *o*, and to date, there have been no reports of a gene encoding a plant nuclear Trx. We report here the presence in pea (*Pisum sativum*) mitochondria and nuclei of a Trx isoform (PsTrx*o*1) that seems to belong to the Trx *o* group, although it differs from this Trx type by its absence of introns in the genomic sequence. Western-blot analysis with isolated mitochondria and nuclei, immunogold labeling, and green fluorescent protein fusion constructs all indicated that PsTrx*o*1 is present in both cell compartments. Moreover, the identification by tandem mass spectrometry of the native mitochondrial Trx after gel filtration using the fast-protein liquid chromatography system of highly purified mitochondria and the in vitro uptake assay into isolated mitochondria also corroborated a mitochondrial location for this protein. The recombinant PsTrx*o*1 protein has been shown to be reduced more effectively by the *Saccharomyces cerevisiae* mitochondrial Trx reductase Trr2 than by the wheat (*Triticum aestivum*) cytoplasmic reduced nicotinamide adenine dinucleotide phosphate-dependent Trx reductase. PsTrx*o*1 was able to activate alternative oxidase, and it was shown to interact with a number of mitochondrial proteins, including peroxiredoxin and enzymes mainly involved in the photorespiratory process.

Thioredoxins (Trxs) are ubiquitous small proteins with a molecular mass around 12 to 14 kD involved in the reduction of disulfide bonds of other proteins. Trxs contain a conserved active site WCG/PPC and a low redox potential that confers reductive properties, being able to regulate specifically target proteins (Laloi et al., 2001). Animals contain only two types of Trxs, one cytoplasmic and one mitochondrial, both well studied and involved in numerous regulatory mechanisms, such as the reduction of peroxiredoxins (Prxs), activation of transcription factors, and signaling of apoptosis (Arner and Holmgren, 2000). Plants contain several nuclear genes encoding Trx proteins (Gelhaye et al., 2005). In Arabidopsis (*Arabidopsis thaliana*), at least 20 *Trx* genes have been reported (Meyer et al., 2002), classified into six families, *Trx f*, *Trx h*, *Trx o*, *Trx m*, *Trx x*, and *Trx y*, and a novel *Trx s*, recently described in *Medicago truncatula* that expands this family of proteins in legumes (Alkhalfioui et al., 2008). The subcellular location of the different Trxs showed that the chloroplast contains Trx *f*, *m*, *x*, and *y* and the atypical form CDSP32 (Collin et al., 2003). A *Trx o* was shown to encode a mitochondrial protein using western-blot and in vitro uptake assays (Laloi et al., 2001), and an additional mitochondrial *h*-type Trx was discovered in poplar (*Populus trichocarpa*; PtTrx*h*2; Gelhaye et al., 2004).

Chloroplastic Trxs are reduced by ferredoxin in a reaction catalyzed by the ferredoxin-Trx reductase, while the cytoplasmic and mitochondrial Trxs are reduced by NADPH Trx reductase (NADPH/TR; Schürman and Jacquot, 2000). The chloroplastic and cytoplasmic Trxs have been widely studied, being implicated in the regulation of the Benson-Calvin

cycle enzymes (Trxs *f*), modulation of the NADP-malate dehydrogenase (Trxs *m*), and redox control of cell proliferation, response to pathogens, and oxidative stress among others (Trxs *h*; López Jaramillo et al., 1997; Reche et al., 1997; Gelhaye et al., 2005).

Our understanding of mitochondrial Trxs in plants is limited; they have been described to be involved in redox regulation, and the alternative oxidase has been shown to be a specific target (Gelhaye et al., 2004). Among possible functions, the detoxification of reactive oxygen species via a mitochondrial Prx has been proposed. PrxIIF has been identified in the genome of Arabidopsis as the only Prx that is targeted to the plant mitochondria (Finkemeier et al., 2005), the poplar and pea (*Pisum sativum*) mitochondrial PrxIIF have been characterized, and a redox-dependent interaction between pea PrxIIF and mitochondrial Trx was recently demonstrated (Gama et al., 2007; Barranco-Medina et al., 2008).

The presence of Trx in the nucleus has been described in animal systems, but only under certain stress conditions where a translocation of cytoplasmic Trx to the nuclear compartment occurred (Hirota et al., 1997; Arai et al., 2006). Some biological functions described for Trxs in this compartment included the supply of reducing equivalents to ribonucleotide reductases and Prx (Laurent et al., 1964; Chae et al., 1994), the regulation of activity of several transcription factors (Hirota et al., 1997), and the control of apoptosis signal-regulated kinase 1 activity (Saitoh et al., 1998). In plants, apart from its cytoplasmic location, a Trx *h* has been described to accumulate in the nucleus of aleurone and scutellum cells of wheat (*Triticum aestivum*) seeds, a location associated with the oxidative stress during germination (Serrato et al., 2001; Serrato and Cejudo, 2003). Unlike the nucleoredoxins, the animal and plant Trxs accumulate in the nucleus, do not have a signal peptide characteristic of this subcellular location, and the presence of Trx in the nucleus of leguminous plants has not been described.

This article presents the isolation from pea leaves of a cDNA encoding a novel mitochondrial Trx *o* protein. Activity assays, subcellular localizations using western-blot analysis, immunolocalization, in vitro import assays, and GFP tagging indicate a mitochondrial and nuclear localization. An affinity protein-protein interaction assay revealed interactions with a variety of mitochondrial proteins, and the ability to regulate mitochondrial alternative oxidase (AOX) is also shown.

RESULTS

Cloning of a *PsTrxo1* and Analysis of Its Amino Acid Sequence

The cDNA sequence encoding the pea Trx, named *PsTrxo1*, comprised 546 bp with a predicted open reading frame of 181 amino acids (Fig. 1A), a calculated molecular mass of 20.1 kD, and a pI of 9.58. Subcellular prediction programs predict a mitochondrial targeting peptide of 69 amino acids to be cleaved upon import to produce a protein with a calculated mass of 12.6 kD and a pI of 6.3. This N-terminal domain is characteristic of mitochondrial targeting sequences particularly enriched in Ser (Glaser et al., 1998; Schneider et al., 1998). The mature protein contains the classic sequence WCGPC in the active site and displays high sequence similarity to Arabidopsis Trxo1 (60%; Laloi et al., 2001) compared with poplar Trx*h*2 (28%; Gelhaye et al., 2002; Fig. 1A).

An analysis of genomic DNA revealed the absence of introns in the sequence, and to our knowledge, all other *Trx* genes characterized in plants contain at least one intron. Comparison of protein sequences with all Arabidopsis Trxs and other Trxs *o* of different plants as described by Laloi et al. (2001) resulted in the protein being classified as a Trx *o* (Fig. 1B). Database searches revealed the existence of several ESTs with high similarity in the protein sequences (more than 80%), indicating a similar type of Trx in other legumes.

Activity of Recombinant PsTrxo1 in Vitro

Recombinant PsTrxo1 protein was able to reduce the insulin-disulfide bridges using dithiothreitol (DTT) as reductant. The in vivo electron donor for the cytoplasmic and mitochondrial Trxs is the NADPH-dependent Trx reductase (Pedrajas et al., 2000). To check if this system is operating for PsTrxo1, we analyzed the in vitro activity of PsTrxo1 protein using two Trx reductases (wheat cytoplasmic NADPH/TR and *Saccharomyces* mitochondrial Trr2) in the insulin reduction assay (Fig. 2). PsTrxo1 reduced insulin disulfide in a NADPH-dependent process in vitro with both reductases, but the mitochondrial Trr2 was more efficient, demonstrating that this protein can form an active Trx system in this organelle.

Subcellular Localization of PsTrxo1

Four sets of experiments were undertaken to determine the subcellular localization of PsTrxo1: western blot, immunogold labeling (Fig. 3), import assay of the precursor protein to mitochondria and chloroplasts, and GFP transient expression (Fig. 4). Additionally, using purified mitochondria, the partial purification of the native PsTrx protein was carried out by chromatography techniques (Fig. 5).

An antibody directed against a peptide of PsTrxo1, shown in Figure 1A, was used to analyze subcellular fractions of pea leaves. The anti-PsTrxo1 recognized the PsTrxo1 recombinant protein, and a band with an apparent molecular mass similar to that predicted (i.e. 12.6 kD) was detected in the mitochondrial fraction but not in the cytoplasmic and chloroplastic fractions (Fig. 3A). The absence of the 12.6-kD band in these latter fractions demonstrates that anti-PsTrxo1 antibody does not cross-react with cytoplasmic or chloroplastic Trxs. Nuclei were purified and analyzed by SDS-PAGE and tandem mass spectrometry (MS/MS) sequencing of the resulting proteins, finding as major

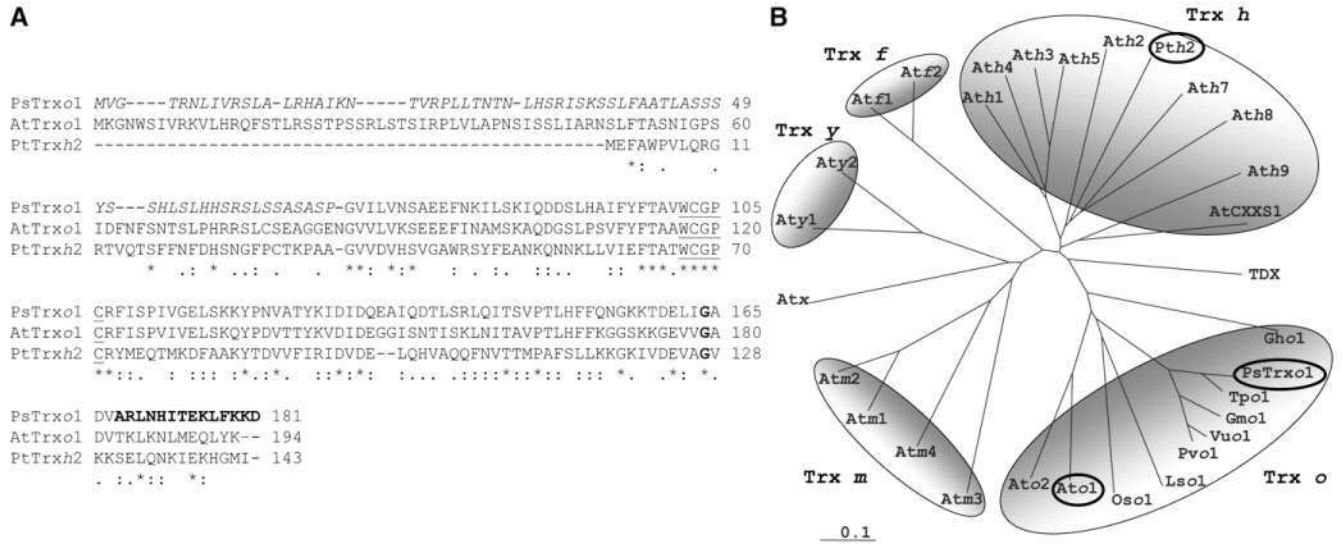


Figure 1. Amino acid sequence analysis of PsTrx*o*1. A, Sequence alignment of pea PsTrx*o*1, Arabidopsis AtTrx*o*1, and poplar PtTrx*h*2 using the ClustalW software. Catalytic sites are underlined. The transit peptide predicted by the MitoProt program in PsTrx*o*1 is shown in italics, and the anti-PsTrx*o*1 antibody produced against the peptide is shown in boldface. B, Phylogenetic tree of Arabidopsis Trxs and Trx *o* homologs from selected plant species (TreeView program). Protein sequences were obtained from the database or deduced from cDNA or EST sequences. The three described mitochondrial Trxs are circled.

proteins the histone H2A, histone H3, and histone H4 and some chloroplast proteins that represented minor contaminants (data not shown). In these nuclei, the antibody specifically detected a major band of an apparent molecular mass of 20.6 kD (Fig. 3A), corresponding to the PsTrx*o*1 protein translated from the cDNA outlined above (Fig. 1A), without any removal of the mitochondrial N-terminal targeting signal.

Immunogold Labeling Studies

To confirm the dual localization of PsTrx*o*1 in the pea leaves, an immunogold labeling study using anti-PsTrx*o*1 was performed. The results displayed high signal intensities in the nuclear heterochromatin and a signal of lower but high magnitude in mitochondria (Fig. 3B). A very low signal corresponding to chloroplasts and cytoplasm was also detected, with no signal detected from the cell wall or vacuole (data not shown). The intensities of the signals obtained from nuclei and mitochondria are in agreement with the western-blot analysis, where the intensity of the reaction with isolated nuclei was stronger. The quantification of this signal was carried out in 20 cells and is presented in the table in Figure 3B.

Import Assays

The mitochondrial nature of the protein was also verified by performing import assays to both pea chloroplasts and potato (*Solanum tuberosum*) mitochondria of PsTrx*o*1 precursor using Rubisco small subunit and AOX as chloroplastic and mitochondrial control proteins, respectively. When PsTrx*o*1 was translated in vitro, the product was expressed as a protein of 20.1 kD molecular mass (Fig. 4A). After incubation of PsTrx*o*1 precursor with mitochondria, a band of around 12.6 kD appeared, whereas no processed band was detected in the case of chloroplasts. This band is resistant to proteinase K, and the import was abolished by the addition of valinomycin, which dissipates the inner membrane potential. The mature PsTrx*o*1 protein obtained after in vitro import corresponds to the 12.6-kD protein immunodetected in pea mitochondria.

In Vivo Targeting Ability of PsTrx*o*1-GFP

To further analyze the targeting ability of PsTrx*o*1, a construct containing a translational fusion of its open reading frame to the GFP reporter gene under the

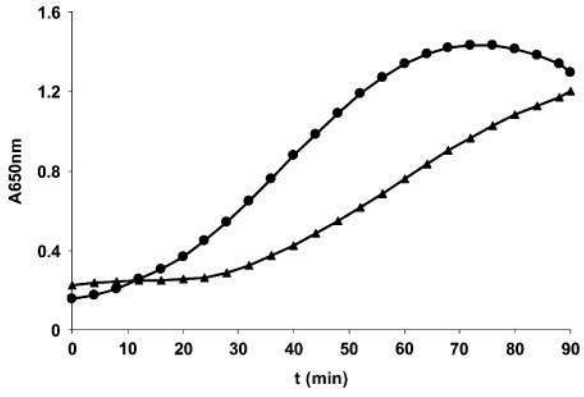
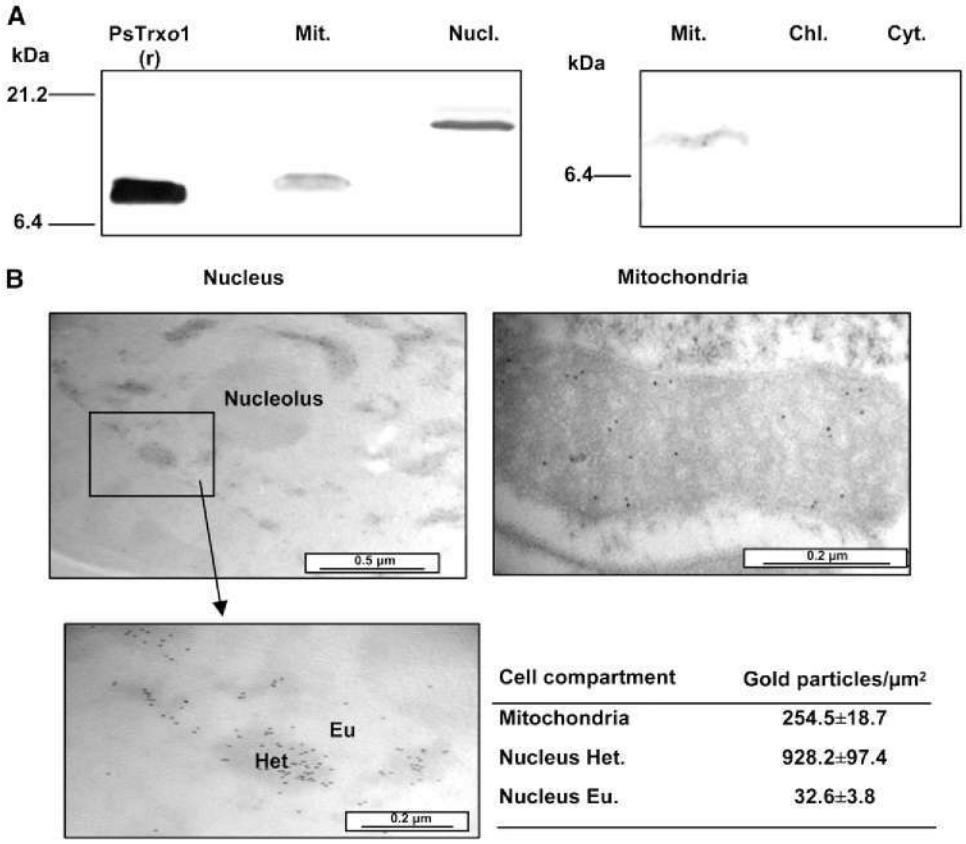


Figure 2. Activity of recombinant PsTrx*o*1 determined by the insulin-disulfide reduction assay. The reduction of insulin was measured by the increase in turbidity at 650 nm for 1 h at 30°C. Triangles, wheat NADPH/TR; circles, *S. cerevisiae* Trr2.



Cell compartment	Gold particles/µm²
Mitochondria	254.5±18.7
Nucleus Het.	928.2±97.4
Nucleus Eu.	32.6±3.8

Figure 3. Subcellular location of PsTrxo1 in pea leaves. A, Western-blot analysis of recombinant PsTrxo1 (r), purified mitochondria (Mit.), nuclei (Nucl.), chloroplasts (Chl.), and cytoplasm (Cyt.) from pea leaves using the antibody generated against the peptide of the PsTrxo1 amino acid sequence indicated in Figure 1A. B, Immunolocalization of PsTrxo1 in pea leaves by transmission electronic microscopy. Left, Nonstained nucleus with detail below; right, mitochondria stained with lead citrate and uranyl acetate. The table shows the gold particles found in each cellular compartment. Eu., Euchromatin; Het., heterochomatin.

control of the 35S promoter was used to bombard onion (*Allium cepa*) epidermal layers. As shown in Figure 4B, panels 1 and 5, the fluorescence emitted by the GFP reporter was targeted to the nucleus of bombarded cells and GFP signal was also detected in mitochondria, as shown by the merged fluorescences of GFP and MitoTracker (Fig. 4B3), further corroborating the presence of PsTrxo1 in both compartments. In contrast, the GFP fluorescence was distributed throughout the cells when the onion epidermal layers were bombarded with the *GFP* gene under the control of the 35S promoter (Fig. 4B9). DNA staining with 4′,6-diamidine-2-phenylindole (DAPI) was used as a control for nuclear location, and the corresponding blue fluorescence was detected in all cells with the exception of those where the presence of the PsTrxo1-GFP protein saturated the nucleus, making difficult the entrance of DAPI (Fig. 4B, panels 6 and 7).

Identification of Native PsTrxo1 in Pea Leaf Mitochondria

Detection of an organelle-specific protein requires the isolation of a great quantity of highly pure organelles, so intact functional mitochondria were obtained following a double gradient isolation method using optimum centrifugation techniques and washing cycles. The maximum obtained degree of chloroplastic, peroxisomal, and cytoplasmic contamination was about 0.01%, 0.07%, and 0.04%, as judged by NADP-dependent glyceraldehyde 3-phosphate dehydrogenase, catalase, and lactate dehydrogenase activities, respectively, and the intactness of the outer mitochondrial membrane was 85% to 90%.

Mitochondria isolated from pea leaves exhibited Trx activity measured as the capacity of induction of the NADP-malate dehydrogenase (MDH; specific activity of about 30–35 units mg^{-1} protein). Twenty-five mitochondria isolation experiments of 50 g of pea leaves each were used for the partial purification of Trx to demonstrate its presence in this organelle. Three milligrams of mitochondrial proteins was subjected to FPLC on Superdex 200 (Fig. 5A). The detection of Trx signal by dot blot analysis of all the fractions eluted allowed us to concentrate the positive fractions (16.8–18.4 mL) of low molecular mass proteins. This fraction also presented Trx activity (450–475 units mg^{-1} protein) and was subjected to SDS-PAGE (Fig. 5B). The analysis by western blot of the concentrated low molecular mass fractions obtained after the FPLC fractionation showed the presence of a unique band recognized by the PsTrxo1 antibody (Fig. 5C) and allowed the identification of a protein band stained with PageBlue with a similar molecular mass to the recombinant PsTrxo1 (Fig. 5B, lane 2, arrow). MS/MS sequencing of the electrophoretic band resulted in two characteristic peptides, IDIDQEAIQDTLSR and KTDELIGADVAR, both contained in the sequence of

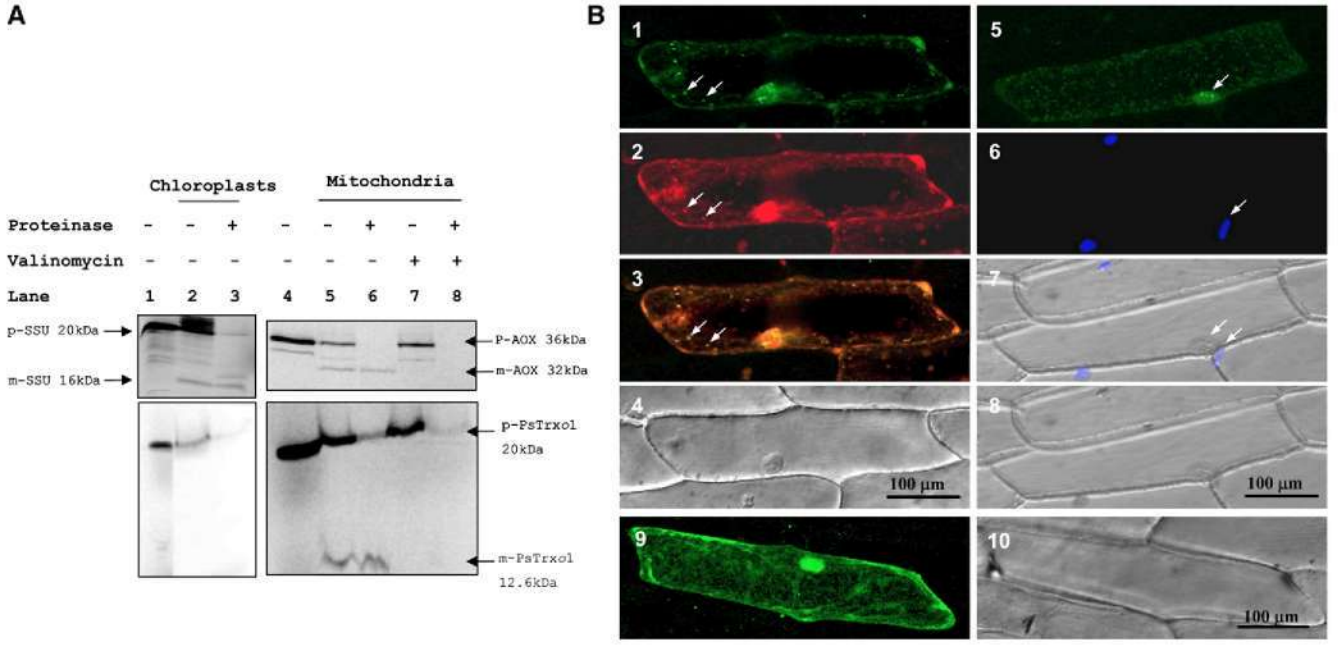


Figure 4. Targeting of PsTrx*o*1 protein. A, Import of precursor PsTrx*o*1-35S into pea chloroplasts and potato mitochondria. Soybean AOX and pea small subunit of Rubisco (SSU) were used as controls. Lanes 1 and 4, Precursor protein alone; lane 2, precursor protein incubated with isolated chloroplasts; lane 3, as for lane 2 but with the addition of thermolysin after the import; lane 5, precursor protein incubated with isolated mitochondria; lane 6, as for lane 5 but with the addition of proteinase K after the import; lane 7, as for lane 5 but with the addition of valinomycin before the import; lane 8, as for lane 7 but with the addition of proteinase K after the import. Migration of the precursor (p) and mature (m) polypeptides is indicated. B, Subcellular location of PsTrx*o*1 protein in onion epidermal cells visualized by confocal microscopy. Epidermal onion layers were transiently transformed with the *PsTrxo1* gene fused to *GFP* (panels 1–8) under the control of the 35S cauliflower mosaic virus promoter. After incubation for 24 h, cells were observed with a confocal microscope for emission spectrum of the GFP (panels 1 and 5). As a control for location, MitoTracker (panel 2) and DAPI staining (panel 6) as well as Nomarski observations (panels 4 and 8) were carried out. Panel 3 depicts the merged images from panels 1 and 2, and panel 7 depicts the merged images from panels 6 and 8. Arrows in panels 1 to 3 indicate mitochondria, and arrows in panels 5 to 7 indicate nuclei. As a control, the transformed cells with the reporter gene GFP (panels 9 and 10) are shown.

the recombinant PsTrx*o*1 (Fig. 1A). This result indicated that mitochondria from pea leaves contain at least one Trx, being to our knowledge the first native Trx partially sequenced and identified by MS/MS from SDS-PAGE of plant mitochondria.

Target Proteins of the Mitochondrial PsTrx*o*1

The mutant PsTrx*o*1C37S was produced in *Escherichia coli* and used for the identification of the target mitochondrial proteins of PsTrx*o*1. Affinity chromatography on a His-binding coaffinity resin column grafted with a monocysteinic Trx mutant was used to identify the potential targets of PsTrx*o*1. When the less reactive Cys of the Trx active site is replaced by a Ser, the heterodisulfide formed by the most reactive Cys and the oxidized target is stabilized and the protein is trapped. MS was used to identify the major spots of soluble mitochondrial proteins trapped in this heterodisulfide form. In a set of experiments from SDS-PAGE of the target proteins in nonreducing and reducing conditions, four enzymes were found as targets of PsTrx*o*1: Gly dehydrogenase components P and T, the ATP synthase subunit α, and the short-chain alcohol dehydrogenase CPRD12. Two-dimensional electrophoresis of the target proteins in the same conditions led us to the identification of five possible new target proteins (Table I): thiosulfate sulfurtransferase (TST) or 3-mercaptopyruvate sulfurtransferase, elongation factor Tu, Ser hydroxymethyl transferase (SHM2), adenylate kinase, and Prx. The ATP synthase α-chain and component P of Gly dehydrogenase were identified again by MS/MS analysis. Apart from all of the described possible targets of PsTrx*o*1 and using this proteomic approach, homoserine dehydrogenase, catalase, and glycolate oxidase were found as contaminants of chloroplast/cytoplasm and peroxisomes in the mitochondrial fractions.

AOX Reduction

AOX was analyzed by western blot as a mitochondrial potential target of PsTrx*o*1. The detection of oxidized AOX dimers and reduced monomers was performed using recombinant PsTrx*o*1-treated or untreated pea leaf mitochondria (Fig. 6A). PsTrx*o*1 re-

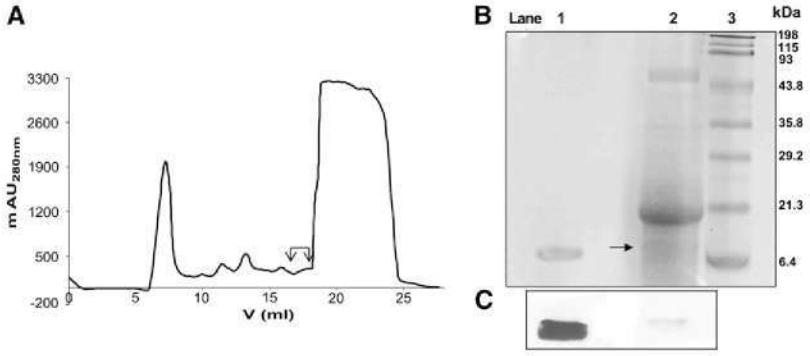


Figure 5. Identification of the native mitochondrial PsTrx*o*1. A, Superdex 200 filtration chromatography of purified mitochondrial proteins. B and C, SDS-PAGE (B) and western-blot analysis (C) of recombinant PsTrx*o*1 (lane 1) and concentrated low molecular mass fractions indicated between arrows in A (lane 2). Molecular mass markers are shown in lane 3.

duced the inactive AOX dimers to active monomers (Fig. 6A, lane 1), suggesting that this mitochondrial Trx could be responsible for the activation of AOX. To test for activation of AOX, oxygen evolution was followed for measuring the AOX respiration pathway using previously oxidized pea and soybean (*Glycine max*) mitochondria, presenting around 0.95% and 8% of respiration state 3, respectively. In both of them, the activity of AOX was monitored in the presence and absence of the Trx system (NADPH/Trr2/PsTrx*o*1). A typical experiment of oxygen uptake by pea mitochondria is shown in Figure 6B (the uptake by soybean mitochondria is presented in Supplemental Fig. S1). Pea mitochondria were oxidized with 10 mM diamide, and respiration was followed in a Suc-depleted medium using NADH as a respiratory substrate. Addition of pyruvate (1 mM) produced a very low activation of the mixothiazol-insensitive respiration, and addition of the PsTrx*o*1, previously reduced by NADPH/Trr2, enhanced respiration by the alternative pathway (Fig. 6B3). A similar behavior was found for the soybean oxidase (Supplemental Fig. S1). However, the activation of pea AOX was higher (7.2-fold) than that of the soybean oxidase (1.3-fold), showing that AOX activity in vivo could be regulated by PsTrx*o*1. In soybean, the activation of the AOX by the Trx system was lower than with DTT (2.5-fold), but pea AOX activation by this system occurred at a higher rate than with DTT (4.8-fold; Fig. 6B2), corroborating again that the system NADPH/TR could be the natural reductant of PsTrx*o*1 in pea mitochondria. The addition of only NADPH/Trr2 (Fig. 6B4) did not enhance the AOX activity, indicating that the presence of PsTrx*o*1 is necessary for the regulation. The addition of PsTrx*o*1 alone did not modify the oxygen uptake (Supplemental Fig. S1, panel 5).

Table I. *Potential Trx target proteins of plant mitochondria identified by affinity chromatography using immobilized Trx resin*

Sequence[a]	Predicted Protein	EMBL Accession No.
VWVLDGGLPR	Thiosulfate sulfurtransferase (TST; *Datisca glomerata*)	AAD19957
	3-Mercaptopyruvate sulfurtransferase (*Arabidopsis thaliana*)	CAB64716
VVDASWYMPNEQR	Unknown protein with a TST region (*Picea sitchensis*)	ABK24999
VAIITGGASGIGEATAR	Short-chain alcohol dehydrogenase CPRD12, drought inducible (*Vigna unguiculata*)	BAA13541
VNCVSPYLVATPLAK		
HTAFFSNYRPQFYLR	Mitochondrial elongation factor Tu (*Arabidopsis thaliana*)	CAA61511
ILDQGQAGDNVGLLLR	Unknown protein with an elongation factor Tu region (*Vitis vivifera*)	CAO22472
ITNFYTNFQVDEIGR	Mitochondrial ATP synthase subunit α (*Pisum sativum*)	CAA28964
EAFPGDVFYLHSR		
VYGLNEIQAGELVEFASGVK		
NTVPGDVSAMVPGGIR	Mitochondrial Ser hydroxymethyltransferase (*Pisum sativum*)	AAA33687
GVNGTVAHEFIIDLR	Mitochondrial Gly dehydrogenase P-protein (*Pisum sativum*)	CAA38252
ESPYLTHPIFNTYQTEHELLR		
VGFISSGPPPR	Mitochondrial Gly dehydrogenase T-protein (*Pisum sativum*)	CAA52800
RAEGGFLGADVILK		
SLLALQGPLAAPVLQHLK		
TGYTGEDGFEISVPSEHGVELAK		
VVIFGLPGAYTGVCSSK	Mitochondrial peroxiredoxin (*Pisum sativum*)	CAG30523
VLNFAIDDAILEER	Adenylate kinase (*Vitis vinifera*)	CAO46644
HESTVGAGLPVIASLNR	Homoserine dehydrogenase (*Glycine max*)	AAZ98830
IAVQSGAAGIIVSNHGAR	Glycolate oxidase (*Glycine max*)	BAG09382
SIWVSYWSQADR	Catalase (*Pisum sativum*)	CAA42736

[a]Obtained tryptic peptide sequences determined by collision-induced dissociation-MS/MS of doubly or triply charged ions generated by in-gel tryptic digestion of protein spots as specified in "Materials and Methods."

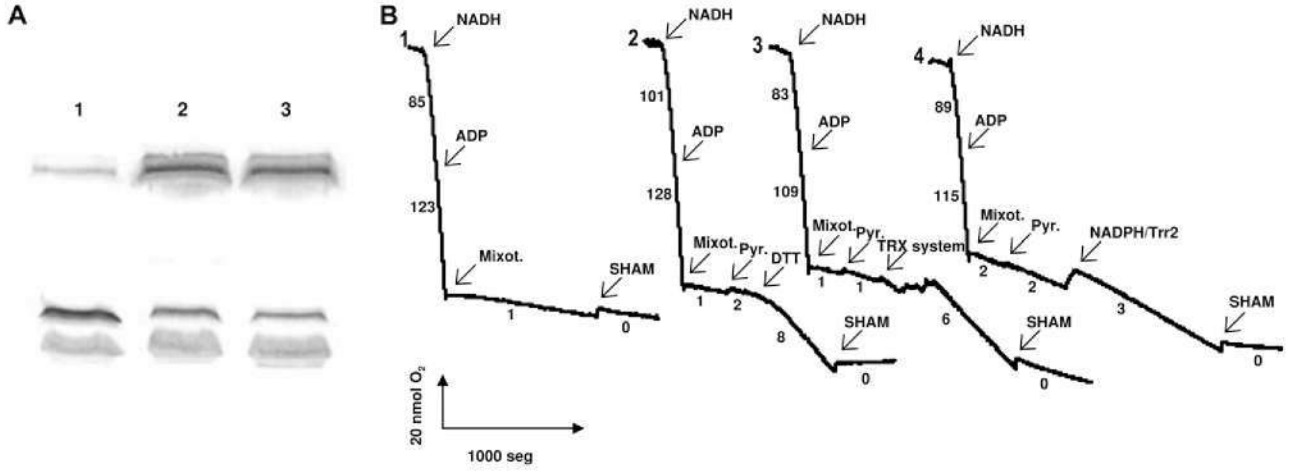


Figure 6. Regulation of AOX by PsTrx*o*1. A, AOX reduction. Lane 1, Pea mitochondria were incubated with the NADPH/Trr2/PsTrx*o*1 system and subjected to SDS-PAGE; lane 2, as for lane 1 but without the addition of PsTrx*o*1; lane 3, control mitochondria. AOX dimers and monomers were immunodetected. B, AOX activation. Oxygen uptake by pea mitochondria previously treated with 10 mM diamide was measured in a Suc-depleted medium. Trace 1, NADH (2 mM) oxidation by purified mitochondria; traces 2 to 4, where indicated, different components were added to the oxygen electrode chamber. The Trx system was NADPH/Trr2/PsTrx*o*1 (trace 3). As controls, 5 mM DTT (trace 2) or NADPH/Trr2 (trace 4) was added. Numbers on the traces represent nmol O_2 min^{-1} mg^{-1} protein from one of three experiments. Mixot., Mixothiazol (inhibitor of cytochrome *c* oxidase); Pyr., sodium pyruvate; SHAM, salicylhydroxamic acid (inhibitor of AOX).

DISCUSSION

Presence of PsTrx*o*1 in the Mitochondria

A gene encoding a novel Trx *o* has been identified and characterized in this study; it contained no introns, a novel feature for plant *Trx* genes, which have at least one intron (Meyer et al., 2002). Genome-wide analysis of intronless genes in rice (*Oryza sativa*) and Arabidopsis (Jain et al., 2008) identified genes encoding Trx reductases in both species and, only in rice, one Prx type 2 and one protein of the Trx family. Phylogenetic analysis grouped this pea Trx with the *o* type; thus, it is called PsTrx*o*1, indicating that although the gene structure is unique, it is a typical Trx protein (see below). The highest similarity found was 60% with AtTrx*o*1, indicating that Arabidopsis does not likely contain a Trx that is orthologous, suggesting that this gene has arisen recently in evolutionary terms. The lack of introns suggests that it may have arisen via retrotransposition, a well-described mechanism to explain gene duplication (Yu et al., 2007; O'Toole et al., 2008). Analysis of ESTs from a variety of plants identified cDNAs encoding proteins with higher similarity with PsTrx*o*1 compared with *Trx* genes from Arabidopsis, all of them belonging to the family Leguminosae (Fabaceae) that split from other angiosperms about 60 million years ago (Soltis et al., 2000). This suggests that this type of Trx gene may be restricted to legumes. The recombinant protein produced in *E. coli* verified Trx activity, being able to reduce insulin in an in vitro assay using two different Trx reductases and NADPH, suggesting that this Trx has the ability to play a role in the redox balance in mitochondria.

The analysis of the amino acid sequence of PsTrx*o*1 revealed the presence of a transit peptide that could direct the protein to mitochondria. A mitochondrial localization was confirmed by western-blot analysis, in vitro import assays, transient expression analysis of GFP fusions, and immunogold labeling. Although the presence of Trx and Trx reductase activities has been detected in plant mitochondria (Marcus et al., 1991; Konrad et al., 1996; Banze and Follmann, 2000), the native proteins responsible for these activities have not been identified and characterized. Molecular filtration chromatography by FPLC of solubilized mitochondrial proteins resulted in the identification and sequencing of peptides contained in the PsTrx*o*1 sequence, confirming that PsTrx*o*1 was responsible for the Trx activity detected with isolated pea mitochondria. This not only confirmed a mitochondrial localization for PsTrx*o*1 but also indicated that it was the only Trx identified in pea that coincided with the pea Trx activity.

Previous studies have identified potential mitochondrial targets but using cytoplasmic or chloroplastic forms of Trx (Balmer et al., 2004). In this study, we used the mitochondrial PsTrx*o*1C37S and identified nine potential mitochondrial Trx targets. An analysis of the proteins in mitochondria that are susceptible to disulfide bonding in Arabidopsis identified AOX, subunits of the ATP synthase, elongation factor Tu, and a range of dehydrogenases. Among the PsTrx*o*1-linked proteins, two proteins of the Gly decarboxylase complex, components P and T, and another important enzyme in photorespiration, SHMT, have been identified. The regulation of these two key enzymes implies the

involvement of mitochondrial Trx in the regulation of the photorespiratory cycle in mitochondria. The activity of the Gly cleavage complex has been described to depend on the presence of DTT in the reaction medium, so Trx could be the natural reductant needed for the activity (Walker and Oliver, 1986). This enzyme involved in the photorespiratory pathway appears particularly susceptible to oxidative damage induced by drought and chilling (Taylor et al., 2002). Similarly, SHMT activity is a key enzyme in the photorespiratory event, and the *shm1-1* mutant of Arabidopsis, defective in SHMT activity, has been shown to display a lethal photorespiratory phenotype when grown at ambient CO_2 (Voll et al., 2006). In this context, it would be very interesting to analyze the role of PsTrx*o*1 in the photorespiratory events in response to oxidative damage situations such as those induced by NaCl and water stress.

Another Trx-linked protein found is the α-subunit of the mitochondrial ATP synthase, which links Trx with the control of ATP synthesis, in a similar manner to the control exerted by chloroplastic Trx *f* as the physiological reductant for the chloroplast ATP synthase (H^+-translocating ATPase) from spinach (*Spinacia oleracea*; Schwarz et al., 1997). Other studies have shown the activation of chloroplastic ATP synthase by Trx (Malyan and Allison, 2002), which accelerated the DTT-dependent activation of CF_1 (Stump et al., 1999). Another protein involved in the transformation of ATP into ADP is adenylate kinase, which has been shown to be a possible target of the mitochondrial PsTrx*o*1. This mitochondrial protein contains an intramolecular disulfide bond as described by Winger et al. (2007). The location of adenylate kinase in the intermembrane space of mitochondria and the presence of a disulfide relay system for the import of proteins into the mitochondria inner membrane (Bandlow et al., 1998) suggest that PsTrx*o*1, or another mitochondrial Trx, may be located in the intermembrane space and matrix and interact with intermembrane space proteins.

Another target of PsTrx*o*1, elongation factor Tu, promotes the GTP-dependent binding of aminoacyl-tRNA to the ribosome during the elongation of newly synthesized protein. As has been previously reported, the chloroplast elongation factor Tu was identified as a potential Trx target with chloroplasts (Balmer et al., 2004).

The binding between the pea mitochondrial proteins PrxIIF and Trx *o* was recently demonstrated using recombinant proteins and isolated mitochondria by isothermal titration calorimetry, molecular filtration, and coimmunoprecipitation (Barranco-Medina et al., 2008), and the mode of binding is characterized by extremely favorable enthalpy changes, demonstrating a role of pea Trx *o* as a physiological electron donor to PrxIIF.

AOX has been identified as a protein with an intramolecular disulfide bond in Arabidopsis (Winger et al., 2007), but it has not been identified as a Trx target using Trx-linked resins, although its regulation by Trx has been proposed (Gelhaye et al., 2004; Umbach et al., 2006). The fact that AOX was not identified as a target in these assays may be due to the low amount of native protein present in the pea mitochondria. As discussed below, the rate of AOX activity in pea is very low compared with that detected in other species such as soybean. We have shown the reduction of pea mitochondrial AOX homodimers by the recombinant PsTrx*o*1 in vitro, as was described for the poplar mitochondrial Trx PtTrx*h*2 (Gelhaye et al., 2004). Also, the activation of AOX, measured by oxygen consumption by this pathway, was performed by PsTrx*o*1 using a NADPH/TR system, showing the regulation of this enzyme by the mitochondrial Trx. In pea, the alternative respiration pathway has been shown to account for only a very low percentage of the total respiration, in contrast to the relatively higher rate in soybean. In this situation, PsTrx*o*1 without NADPH/TR is not able to reduce AOX and activate the alternative pathway, suggesting NADPH/TR as the possible in vivo reductant of the mitochondrial PsTrx*o*1. Another interesting difference between mitochondria of both species is the higher ability of PsTrx*o*1 to activate AOX in pea mitochondria compared with the soybean organelle, in which DTT is more efficient in the reduction than the Trx system. NADPH/TR/PsTrx*o*1, then, could be a highly effective system in the activation of the AOX pathway in pea. This activation had not been demonstrated for a Trx *o*, and the next step could be the characterization of their in vivo interaction by fluorescence resonance energy transfer. AOX plays an important role in decreasing reactive oxygen species (Maxwell et al., 1999; Yip and Vanlerberghe, 2001), and PsTrx*o*1 could play a major role in the activation of AOX and in the detoxification of reactive oxygen species in mitochondria in normal conditions and under stress situations.

The identification of the peroxisomal proteins catalase and glycolate oxidase as possible Trx targets raises the question of the presence of Trx and its role in this organelle, as described by Balmer et al. (2004) for catalase, possibly extending the role for Trx in the plant cell and consistent with the proposed role of PsTrx*o*1 in the photorespiratory process.

Presence of PsTrx*o*1 in the Nucleus

The transient expression of the PsTrx*o*1-GFP fusion protein in *Allium* epidermal cells allowed us to corroborate the presence of this Trx in the nucleus. The import of proteins into the nucleus is a highly selective process that depends on a nuclear location signal of short stretches of basic amino acids, which are not cleaved after import. However, this is not the case for the mammalian cytoplasmic Trxs, which lack an obvious nuclear location signal (Hirota et al., 2000). No clear nuclear location signal was found within the deduced amino acid sequence of PsTrx*o*1. The analysis of the common nuclear location signals has shown that about 50% of the nuclear proteins contain a bipartite motif comprising two basic amino acids, a spacer region of any 10 to 16 amino acids (nucleoplasmin and influenza

virus BP1 protein, respectively, as examples) or even 37 amino acids (adenovirus DNA-binding protein), followed by a basic cluster in which three of the next five amino acids must be basic (Dingwall and Laskey, 1991). PsTrx*o*1 sequence presents in the C terminus one of these bipartite motifs (amino acids 157–181), which could be responsible for the direction of the protein to the nucleus. Using the transient expression assay, western-blot analysis, and immunogold labeling, clear evidence that the PsTrx*o*1 protein localizes to the nucleus was shown. In plants, Trxs *h*, typically located in cytoplasm, have been reported to accumulate in the nucleus of aleurone and scutellum cells during germination, a location associated with oxidative stress (Serrato and Cejudo, 2003), similar to that described for the nuclear mammalian Trxs (Hirota et al., 1999, 2000; Wei et al., 2000). However, in this study, PsTrx*o*1 is located in the nucleus under normal conditions, suggesting a role that may not be linked only to the stress response and extending the roles of these proteins in other processes in plants. Nuclear location is consistent with the large body of evidence indicating that members of the mammalian Trx family play key roles in transcriptional regulation; moreover, the presence of PsTrx*o*1 in the heterochromatin could be related to the described involvement of the mammalian mitochondrial/peroxisomal Prx PRDX5 in protecting the genome against oxidation and in the control of transcription of noncoding DNA (Kropotov et al., 2006).

CONCLUSION

A new Trx is present in leaves from pea plants. This Trx (PsTrx*o*1) has sequence similarity to the AtTrx*o*1, but as a different feature among Trxs, the *PsTrxo1* gene is characterized by the absence of introns. Interestingly, PsTrx*o*1 is present not only in mitochondria but also in nuclei. The native mitochondrial Trx has been partially sequenced. The recombinant protein presented a higher capacity of insulin reduction with the *S. cerevisiae* mitochondrial Trr2 than with the wheat cytoplasmic NADPH/TR, suggesting that this system could be operating in vivo. PsTrx*o*1 was able to activate the AOX and presented a reduced number of mitochondrial target proteins, including PrxIIF and some photorespiratory enzymes, indicating that this Trx may have an important role in this process.

MATERIALS AND METHODS

Plant Material

Pea (*Pisum sativum*) plants were cultured in a growth chamber, under optimum conditions, for 2 weeks as described by Jiménez et al. (1997).

cDNA Preparation and Genomic DNA Isolation

Total RNA was isolated from 100 mg of young pea leaves using the RNeasy Plant Mini Kit (Qiagen). cDNA was obtained by reverse transcription for PCR using SuperScript II reverse transcriptase (Invitrogen) with an oligo$(dT)_{20}$ as a primer.

Two different methods were used in order to isolate genomic DNA, the Wizard sv Genomic DNA purification system (Promega) and the Nexttec clean-columns (Nexttec), using 1 g and 100 mg of young pea leaves, respectively.

Identification of a Gene Encoding a Trx*o*1

Two primers were constructed based on the sequence of pea ('Lincoln') mRNA for putative mitochondrial Trx (GenBank/EMBL database, accession no. AM235208): primer 1, 5′-ATGGTTGGAACCAGAAATTTGATC-3′, which encodes the peptide sequence MVGTRNLI; and primer 2, 5′-TTAGTCC-TTCTTGAAGAGTTTCTC-3, used as the reverse primer, which encodes the peptide sequence EKLFKKD. PCR was performed at 56°C, and its product was gel purified, cloned in pGEM-T (Promega), and sequenced. The same experiment was performed using genomic DNA to obtain the number and positions of introns in the sequence.

Prediction of Subcellular Localization and Comparison of the Deduced Amino Acid Sequences of PsTrx*o*1 and Other Trxs

MitoProt (prediction of signal peptide; Claros and Vincens, 1996) and Predotar and TargetP (subcellular localization; Emanuelsson et al., 2000; Small et al., 2004), provided by the ExPASy server (http://us.expasy.org), were used for the analysis of the deduced amino acid sequences. The ClustalW program, provided by the European Bioinformatics Institute server (http://www.ebi.ac.uk), was used for alignment. TreeView (version 1.5.2) was employed to create an unrooted phylogenetic tree.

Expression and Purification of Recombinant and Mutant Trxs

A fragment of cDNA encoding the mature protein (amino acids 70–181), without the mitochondrial transit peptide, was obtained by reverse transcription-PCR. This fragment was cloned into the *Nco*I/*Bam*HI sites of the pET3d expression vector (Novagen). The recombinant mutant pea Trx, PsTrx*o*1C37S, was prepared by replacing the second Cys of the active site of the PsTrx*o*1 as reported by Pérez-Pérez et al. (2006) and was cloned into the *Bam*HI site of the pET15b vector, which adds a His tail. *Escherichia coli* BL21 (DE3) strains were transformed with the resulting constructions, and recombinant protein expression was induced by the addition of 1 mM isopropylthio-β-galactoside for 3 h at 37°C.

The purification of the recombinant PsTrx*o*1 was performed by FPLC of the bacterial lysate. The *E. coli* cells were broken with a French press, following by ammonium sulfate precipitation between 40% and 95% (w/v). The pellet was then suspended in buffer (25 mM Tris-HCl, pH 8.0, containing 150 mM NaCl) and chromatographed using a Superdex 75 column. The mutant PsTrx*o*1C37S was purified from cells that were broken with Alumin (Sigma) and sonication, using a Co^{2+} affinity chromatography system following the manufacturer's instructions (Talon Metal Affinity Resin; Clontech).

Preparation of Rabbit PsTrx*o*1 Antiserum

The polyclonal antibody against the C-terminal sequence ARLNHI-TEKLFKKD of mature PsTrx*o*1 was raised in New Zealand White rabbits by Sigma-Genosys.

Cell Fractionation

All steps were carried out at 4°C. Chloroplasts and cytoplasm from pea leaves were isolated as described by Gómez et al. (1999) and Hernández et al. (2000). For the isolation of mitochondria, two different methods were used consecutively. Mitochondria were first isolated from pea leaves as described by Day et al. (1985) and were subjected to a second round of Percoll gradient purification as described by Jiménez et al. (1997) to obtain a highly pure mitochondrial fraction. The integrity of the outer mitochondrial membrane was estimated from the succinate: cytochrome *c* oxidoreductase activity (Hernández et al., 1993) and different marker enzymes for other cell compartment were measured to check the organelle purification level (Jiménez et al., 1997). Around 25 mitochondrial isolation experiments (approximately 1.2 kg of pea leaves) were used for the Trx partial purification. For the isolation of highly pure nuclei, the Plant Nuclei Isolation Extraction Kit (CelLytic PN; Sigma) was used following the manufacturer's instructions.

Trx Activity

Two different methods were used to measure Trx activity. The insulin reduction method is based on the capacity of reduced Trx to reduce oxidized insulin, measuring the increase in turbidity at 650 nm for 1 h at 30°C as described by Martínez-Galisteo et al. (1993). Trx was reduced by DTT or the NADPH/TR system as described by Laloi et al. (2001) using the cytoplasmic NADPH/TR from wheat (*Triticum aestivum*) and the mitochondrial Trr2 from *Saccharomyces cerevisiae*. The second method was based in the induction of NADP-dependent MDH (EC 1.1.1.82; Scheibe et al., 1993). For this, the purification of a chloroplastic NADP-MDH from 500 g of pea leaves was carried out as described by Fickebser and Scheibe (1983) and Reche et al. (1997) using FPLC.

Protein concentration was measured according to the Bradford (1976) protocol.

PAGE and Western-Blot Analysis

Proteins were resolved using SDS-PAGE as described (Laemmli, 1970). Gels were stained with PageBlue (Fermentas). For western-blot analysis, proteins were transferred onto a nitrocellulose membrane using a semidry blotting apparatus (Bio-Rad). Immunoreaction was carried out using rabbit polyclonal antibody against PsTrxo1 diluted 1:2,000 in Tris-buffered saline (TBS) containing 1% (w/v) bovine serum albumin (BSA) and 0.1% (v/v) Tween 20. Goat anti-rabbit antibody conjugated to alkaline phosphatase (Boehringer Mannheim) was used as the secondary antibody. The antigen was detected by colorimetry using nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Roche) following the manufacturer's protocol.

Partial Purification and Identification of the Native Mitochondrial Trx

Purified mitochondria were ruptured by three freeze-thaw cycles, resuspended in 10 mM HEPES-NaOH buffer, pH 7.2, containing 10% (v/v) glycerol, 1 mM $MnCl_2$, 2 mM sodium ascorbate, 14 mM β-mercaptoethanol, 2 mM phenylmethylsulfonyl fluoride, and 0.1% (v/v) 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonic acid (CHAPSO), and incubated on ice for 15 min followed by sonication for 1 min. The solution was concentrated using carboxymethyl cellulose. Three milligrams of the protein solution was then chromatographed on a Superdex 200 FPLC system (Pharmacia) equilibrated in 50 mM Tris-HCl buffer, pH 7.9, containing 150 mM NaCl and 10 mM DTT, at a flow rate of 0.8 mL min^{-1} and room temperature. Fractions were collected and stored at −80°C. Dot-blot assay on these fractions was performed using the antibody generated against a PsTrxo1 peptide. Once identified, the positive low molecular mass fractions were collected and concentrated using Amicon filters (YM3) followed by 10 mM DTT treatment for 15 min. SDS-PAGE and PageBlue protein staining together with western-blot analysis were performed in order to identify the band corresponding to Trx. Once identified, the band of interest was sequenced by MS/MS.

Proteomic Characterization

Proteins of interest were excised from gels and subjected to automated reduction, alkylation, and digestion with sequencing-grade bovine pancreas trypsin (Roche) using a ProGest digestor (Genomic Solutions) following the manufacturer's instructions. The tryptic peptide mixtures were analyzed with an Applied Biosystems Voyager-DE-Pro matrix-assisted laser-desorption ionization time of flight mass spectrometer. For peptide sequencing, the protein digest mixture was subjected to electrospray ionization mass spectrometric analysis (QTrap 2000 [Applied Biosystems] with a nanoelectrospray source [Protana]; Le Blanc et al., 2003). Collision-induced dissociation spectra were interpreted manually or using a licensed version of the MASCOT program (http://www.matrixscience.com) against the SwissProt/TrEMBL database (http://us.expasy.org/sprot/). MS/MS mass tolerance was set to ±0.6 D. Carbamidomethyl Cys and oxidation of Met were fixed and variable modifications, respectively. Amino acid sequence similarity searches were performed using BLAST (Altschul et al., 1997) at http://www.bork.embl.de/j/.

Electron Microscopy and Immunogold Labeling

Leaf samples were fixed and embedded in London Resin White acrylic resin. Ultrathin sections (60–70 nm) were placed on coated nickel grids. Grids were blocked in TBS plus 2.5% (w/v) BSA. They were then incubated for 2 h with anti-PsTrxo1 antibody diluted 1:200 in TBST buffer (TBS + 1% BSA + 0.05% Tween 20). After washing with TBST, the sections were incubated for 1.5 h with the secondary antibody goat anti-rabbit IgG, gold labeled (10 nm; British BioCell International), and diluted 1:50 with TBST. The sections were washed several times in TBST buffer, TBS, and water. Grids were observed with a Philips Tecnai 12 transmission electron microscope. Two different controls were used, one omitting the anti-PsTrxo1 antibody and the other using preimmune serum instead of anti-PsTrxo1.

Import of Radiolabeled Proteins into Isolated Mitochondria and Chloroplasts

Precursor proteins were synthesized from corresponding cDNA clones in pGEM-3Zf+ by an in vitro coupled transcription-translation system with the Promega TnT T7 or SP6 rabbit reticulocyte lysate system in the presence of [^{35}S]Met according to the supplier's instructions. Intact chloroplasts were isolated from 10-d-old pea leaves as described by Bruce et al. (1994) and from potato (*Solanum tuberosum*) tuber mitochondria as described by Glaser et al. (1995). Chloroplast and mitochondria import assays were carried out as described by Waegemann and Soll (1995) and Glaser et al. (1995), respectively. The following precursor proteins were used as controls: soybean (*Glycine max*) AOX (Whelan et al., 1993) and, for pea, the small subunit of Rubisco (Anderson and Smith, 1986).

Location of the PsTrx*o*1-GFP Fusion Protein in Onion Epidermal Cells

To create the translational fusions of the *PsTrxo1* gene to the *GFP* reporter gene, the corresponding complete cDNA including the transit peptide was PCR amplified using the following primers: forward oligo, 5′-CACCATGGTTGGAACCAGA-3′; reverse, 5′-GTCCTTCTTGAAGAGTTTC-3′. The fragment was cloned into the pENTR/D-TOPO vector (Invitrogen) and, following confirmation of sequence fidelity, was subcloned in-frame to the N-terminal *GFP* gene by a recombination reaction into the plasmid pMDC83 containing the cauliflower mosaic virus 35S promoter and a soluble modified green-shifted GFP as described by Curtis and Grossniklaus (2003). The resulting plasmid, pPsTrxo1-GFP, was sequenced to confirm that the site of fusion was in-frame. Primer design and the in vitro clonase recombination reactions were carried out according to the manufacturer's instructions (Invitrogen). As a control, pMDC83 without the PsTrxo1 insert was used.

Constructs were transferred to onion (*Allium cepa*) epidermal cells by biolistic bombardment. Inner epidermal layers of onion bulbs locally purchased were peeled and placed onto Murashige and Skoog agar medium diluted twice as described by Borrell et al. (2002). Particle bombardment was performed with a biolistic helium gun device (PSP-1000; DuPont) basically as described by Díaz et al. (2005). The GFP fluorescence was observed after 24 h of incubation at 22°C in the dark with a confocal ultraspectral microscope (TCS-Sp2-AOBS-UV; Leica). As a control for nuclear location, Nomarski observations were carried out and tissue samples were stained with DAPI (Serva). As a control for mitochondrial location, tissue samples were stained with MitoTracker Red 580 (Invitrogen).

Identification of Trx Target Proteins

Mitochondrial proteins were isolated as described above. For the identification of target mitochondrial proteins, we followed essentially the method described by Pérez-Pérez et al. (2006). Briefly, in each experiment, around 2.6 mg of recombinant protein PsTrxo1C37S was bound to 400 μL of His-binding coaffinity resin (Clontech) and incubated with 5 to 6 mg of mitochondrial proteins for 16 h at 4°C under gentle agitation. Washing and elution with imidazole of intact Trx target disulfide complexes were performed as described by the manufacturer. Two-dimensional nonreducing and reducing SDS-PAGE for optimal overall resolution of Trx and target proteins was performed. Parallel one-dimensional nonreducing and reducing SDS-PAGE was performed in order to increase the resolution of the protein bands to be sequenced as described above.

AOX Reduction and Activity

The reduction of pea AOX by PsTrxo1 was performed by incubation of purified pea mitochondria samples that had undergone three cycles of freeze/

thaw with the NADPH/Trr2/PsTrxo1 system. Mitochondrial proteins were incubated for 45 min at 37°C with 12 μM PsTrxo1, 200 mM mitochondrial Trr2 from *S. cerevisiae*, and 2 mM NADPH before being subjected to nonreductive SDS-PAGE. AOX immunodetection was performed using a monoclonal antibody.

To measure the AOX activity, freshly purified pea and soybean mitochondria isolated as described by Jiménez et al. (1997) and Day et al. (1985), respectively, were incubated with 10 mM diamide for 30 min at 4°C and washed twice. Activation of AOX was measured after incubation of the freshly purified mitochondria with 16 μM PsTrxo1 previously reduced for 45 min in the presence of 16 mM NADPH and 1.6 μM Trr2. The oxygen uptake was measured with a Clark-type O_2 electrode (Rank Brothers) in a Suc-depleted respiration medium (5 mM KH_2PO_4, 10 mM *N*-[Tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid, pH 7.2, 10 mM KCl, and 2 mM Mg_2SO_4) at 25°C using 5 μM mixothiazol and 1 mM salicylhydroxamic acid as inhibitors of the cytochrome and AOX respiration pathways, respectively. Subsequent additions were made as described in Figure 6B.

Sequence data from this article can be found in the GenBank/EMBL databases under the following accession numbers: for *Arabidopsis thaliana*, *Ath2* (S58123), *Ath3* (S58118), *Ath4* (S58119), *Ath5* (S58120), *Ath7* (AAD39316), *Ath8* (AAG52561), *Ath9* (AAG51342), *Atf1* (Q9XFH8), *Atf2* (Q9XFH9), *Atm1* (O48737), *Atm2* (AAF15949), *Atm3* (AAF15950), *Atm4* (Q9SEU6), *Atj1* (Q6NPF9), *Atj2* (Q8L7S9), *Ato1* (AAC12840), *Ato2* (AF396650), *Atx* (AAF15952), AtCXXS1 (Q8GXV2), and TDX (Q7XJ63); for *Populus trichocarpa*, *Pth2* (AF483266). Accession numbers for EST contigs are as follows: *Trifolium pratense*, Tpo1 (BB932971); *Glycine max*, Gmo1 (CO982355); *Vigna unguiculata*, Vuo1 (FG912242); *Phaseolus vulgaris*, Pvo1 (CV538300); *Gossypium hirsutum*, Gho1 (AI725806 and AI729485); *Solanum lycopersicum*, Lso1 (BE462179, AW037483, AW037392, and BE433326); and *Oryza sativa*, Oso1 (C27892 and AU100897).

Supplemental Data

The following material is available in the online version of this article.

Supplemental Figure S1. Soybean AOX activation by PsTrxo1.

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